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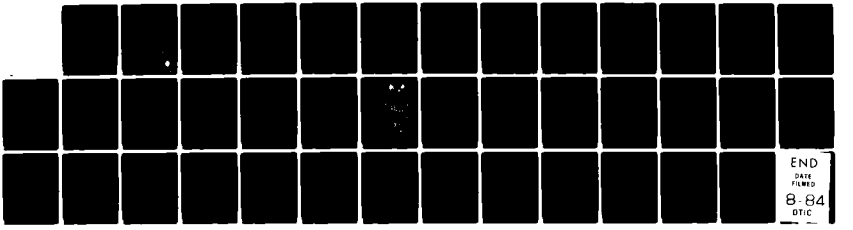

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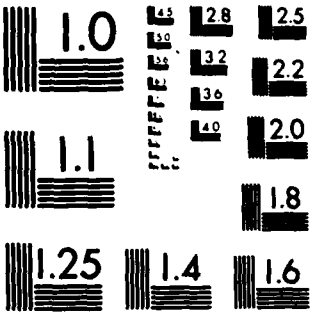

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Report USAFSAM-TR-84-12

EFFECTS OF RADIOFREQUENCY RADIATION ON DIFFERENTIATION OF ERYTHROLEUKEMIC CELLS

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May 1984

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NOTICES

This final report was submitted by the Life Sciences and Electrical Engineering Departments, University of Missouri-Rolla, Rolla, Missouri 65401, under contract F33615-82-K-0634, job order 7757-01-84, with the USAF School of Aerospace Medicine, Aerospace Medical Division, AFSC, Brooks Air Force Base, Texas. Dr. David N. Erwin (USAFSAM/RZP) was the Laboratory Project Scientist-in-Charge.

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The Office of Public Affairs has reviewed this report, and it is releasable to the National Technical Information Service, where it will be available to the general public, including foreign nationals.

This report has been reviewed and is approved for publication.

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A dose-response study was conducted to determine if chemically induced erythroid differentiation of murine erythroleukemic (MEL) cells is affected by continuous-wave radiofrequency (RF) radiation. RF exposures were at 1180 MHz in a specially constructed anechoic chamber equipped with a constant-temperature-air circulator designed to maintain the cell at 37.4°C. Experiments were performed at incident power densities of 5.5, 11, and 22 mW/cm^2, corresponding to SAR levels of 18.5, 37, and 74 W/kg, respectively. Four replicate experiments were conducted at each power level with two irradiated and two control cultures included in each replicate. Cultures were initiated by suspension of MEL cells in growth medium containing the inducer hexamethylene biasacetamide, 3 mM, in 10-cm cellulose nitrate tubes. One day after addition of the inducer, the cultures were placed in the exposure chamber and irradiated for 48 hr. Cells were then counted, resuspended in normal growth medium (no inducer), and incubation continued for 2 additional days. Number of cells undergoing erythroid differentiation was determined by staining with benzidine, a hemoglobin-specific

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reagent. Amount of hemoglobin present in lysates prepared from the differentiated cells was determined by a colorimetric procedure. Irradiated and control cultures were found to grow at the same rate, doubling time of about 16 to 17 hr in the presence of the inducer. Irradiated and control cultures had similar numbers of cells, approximately 65 to 75%, that were benzidine-positive. And, amounts of hemoglobin in the irradiated and control cultures, about 4 to 8% of the soluble protein fraction, were also similar. Results are discussed in relation to proposed RF exposure limits.

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EFFECTS OF RADIOFREQUENCY RADIATION ON DIFFERENTIATION OF ERYTHROLEUKEMIC CELLS

INTRODUCTION

Many recent investigations of radiofrequency (RF) radiation bioeffects have focused on mammalian growth and development. Most of these investigations have involved the whole animal. Nawrot et al. (25), for example, analyzed the offspring of pregnant CD-1 mice subjected to 2.45-GHz irradiation at power densities of 5, 21, and 30 mW/cm^2. Decreased fetal weight followed exposure to the 30 mW/cm^2 field during days 1 to 6 of gestation. Exposures to the same power level during days 6 to 15 of gestation also increased the incidence of fetal malformation. A similar 2.45-GHz study by Berman et al. (2) revealed reduced weight in neonatal offspring of pregnant CD-1 mice following 28-mW/cm^2 irradiation during days 6 to 17 of gestation. Inouye et al. (13) examined day 4 preimplantation embryos flushed from the uterine horns of time-bred CD-1 mice exposed on day 2 or 3 postconception to 2.45-GHz power densities of 9 and 19 mW/cm^2. Exposure of a separate group of pregnant dams to a known heat stress, 38°C ambient temperature, resulted in recovery of fewer embryos and stunted progression from the morula stage to the blastocyst stage of development. The 2.45-GHz irradiation had no effect, however, on quantity or development of preimplantation embryos.

Other frequencies within the RF spectrum have been tested for effects on growth and development. Lary et al. (16) reported teratogenic responses in pregnant Sprague-Dawley rats irradiated with 27.12-MHz waves on day 9 of gestation. In contrast, Smialowicz et al. (31) observed no developmental anomalies in rats subjected to prenatal and postnatal 100-MHz exposures at 46 mW/cm^2.

The thermal response of the experimental subjects to RF irradiation must be considered in assessing the significance of these findings. The fetal weight reductions and malformations observed in three of the studies cited (2, 16, 25) were correlated with significant increases in the body temperature of the irradiated dams. These effects appear due, therefore, to RF-induced hyperthermia and are, in that sense, similar to the teratogenic effects of hyperthermia induced by other, more conventional methods (8). Different findings were obtained in related investigations of RF effects on insect development. Lindauer et al. (18) and Liu et al. (20) described various teratogenic responses of insect pupae to low-level 9-GHz irradiation, effects the investigators suggest are not thermal in origin. This conclusion was based on evidence that equivalent heating of pupae by other means did not induce teratogenesis (18).

Accurate predictions of potential RF bioeffects, including effects on development, require elucidation of the mechanism(s) of RF interaction with biologic systems. One possible mechanism of interaction, proposed by Prohofsky and colleagues (15, 24, 27, 29) might involve absorption of RF protons by resonant modes of deoxyribonucleic acid (DNA). The theoretical analyses of these investigators indicate that RF-induced vibrations of DNA may be localized at junctions between double-stranded and single-stranded regions of the macromolecule (28). The observation of a resonant mode at about 600 MHz in dry fibers of calf thymus DNA (19)

partially supports these predictions and strengthens the possibility of athermal RF effects in vivo. If such vibrations are localized at double-strand and single strand junctions as predicted, both replication and transcription could potentially be affected. It is noteworthy that a 25% inhibition of DNA replication was reported for L1210 cells exposed to 1.0-GHz radiation at 20 mW/cm^2 (4). The developing embryo, differentiating bone marrow, and other tissues characterized by intense replicative and transcriptional activities could be particularly sensitive to localized DNA resonances.

Whole animal experiments are of limited value in studies of cellular and molecular processes of development because of the inability to define or control the complex in vivo environment. Cell systems that proliferate and differentiate in vitro can, however, be used as model systems for investigation of RF effects on developmental processes. One model system commonly used to study development at the cellular level is the murine erythroleukemic (MEL) cell, a cell that can be chemically induced to progress through a sequence of biochemical and cytological events that mimic erythroid differentiation of hemopoietic stem cells (22). These similarities include accumulation of globin messenger ribonucleic acid (mRNA) (33), synthesis of hemoglobin (9), and alteration to a normoblast-like appearance (26).

This investigation assessed effects of RF radiation on erythroid differentiation of MEL cells treated with hexamethylene bisacetamide (HMBA), a potent inducer of differentiation (10, 30). Cultures of HMBA-induced MEL cells were exposed for 2 days to 1180-MHz, continuous-wave (CW) radiation at incident power densities of 5.5, 11, and 22 mW/cm^2. RF exposures were in an anechoic chamber equipped with a constant-temperature air system for maintenance of cultures at 37°C. Control cultures maintained in equivalent temperature water baths were included for comparison. Four replicate experiments were performed at each power density with each replicate including two irradiated and two control cultures. The specific parameters assayed to assess RF effects included rate of cell proliferation, percentage of cells stained by the hemoglobin-specific dye benzidine, and quantity of hemoglobin produced by the differentiating MEL cells. The latter two parameters were used to assess the extent of differentiation. Temperatures of the irradiated and control cultures were both rigorously maintained at 37.4°C to obviate temperature artifacts.

RF EXPOSURE SYSTEM

RF Source and Instrumentation

The RF power source was a CW generator (model 250C, Epsco) equipped with a model M8060H (700-1200 MHz) plug-in oscillator, capable of a nominal power output of 250 W. Power was routed from the RF generator via RF 213/U coaxial cable through a dual-directional coupler (Narda model 3022) to a feedhorn mounted at the top of the exposure chamber. The forward and reflected powers were measured by power meters (model HP 435A, Hewlett-Packard) with power sensing probes (model HP 8482H, Hewlett-Packard) connected to the directional coupler ports. The RF field strength was directly measured with a broadband isotropic radiation monitor (model 8606, Narda) fitted with a 0.3- to 26-GHz probe (model 8623, Narda).

Temperature of the culture medium of the irradiated cell cultures and water bath temperature of the control cell cultures were measured with two noninterfering thermistor probes attached to electronic temperature meters (model 101, Vitek). Outputs from the power meters and the temperature meters were connected to a logging multimeter (model HP 3467A, Hewlett-Packard). Power and temperature readings were logged at 10- or 30-min intervals during the 2-day irradiation period.

Anechoic Chamber and Constant-Temperature-Air Circulator

A specially constructed anechoic chamber (Fig. 1) was used for incubation and RF-irradiation of cultures. The anechoic chamber consisted of a 1.22- x 1.22- x 1.22-m plywood box lined with microwave absorbant material (type AAP-12, Advanced Absorber Products). The corners of the chamber contained 20-cm-thick absorbant material added to further reduce RF leakage. A 11.43- x 22.45-cm feedhorn, a coax-waveguide adapter, was positioned at the top of the chamber, 45 cm above a Styrofoam work deck. A removable access port lined with absorbant material permitted entry to the chamber. The integrity of the absorbant surface was maintained when the access port was in place.

Constant-temperature air was recirculated through the anechoic chamber via a Styrofoam-insulated duct system as shown in Figure 1. Two temperature-sensing thermistors positioned in the air duct supplied feedback information to the temperature control unit. The two output channels of the temperature controller provided power to a preheater, a coiled-wire element, and an auxiliary heater, a 60-W light bulb. The control unit and associated heating devices maintained temperature of the air flow within $\pm$ 0.2^{o}C for extended durations.

Tubes containing the irradiated cell cultures were positioned in a holder specially constructed of 1-mm-thick sheets of balsa wood (Fig. 2). The design of the holder, suggested by Dr. O. Sitton (personal communication, 1983), forced the air to flow over the culture tubes in a crisscrossed fashion, minimizing thermal gradients in the air. The tube holder was in turn placed in a 2-mm depression in the air duct. The air duct and tube holder were oriented to maintain the two RF-exposed tubes parallel to the E-field and equidistant from the center of the feedhorn aperture (Fig. 3). As shown in the figure, the noninterfering probe of a Vitek meter was inserted into one of the two tubes to permit continuous recording of culture temperature during irradiation.

TECHNICAL APPROACH

Chemicals and Culture Supplies

Powdered Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Flow Laboratories. Plastic culture dishes and tubes were from Lux. Hexamethylene bisacetamide (HMBA), benzidine, and mouse hemoglobin were products of Sigma. All other chemicals were reagent grade.

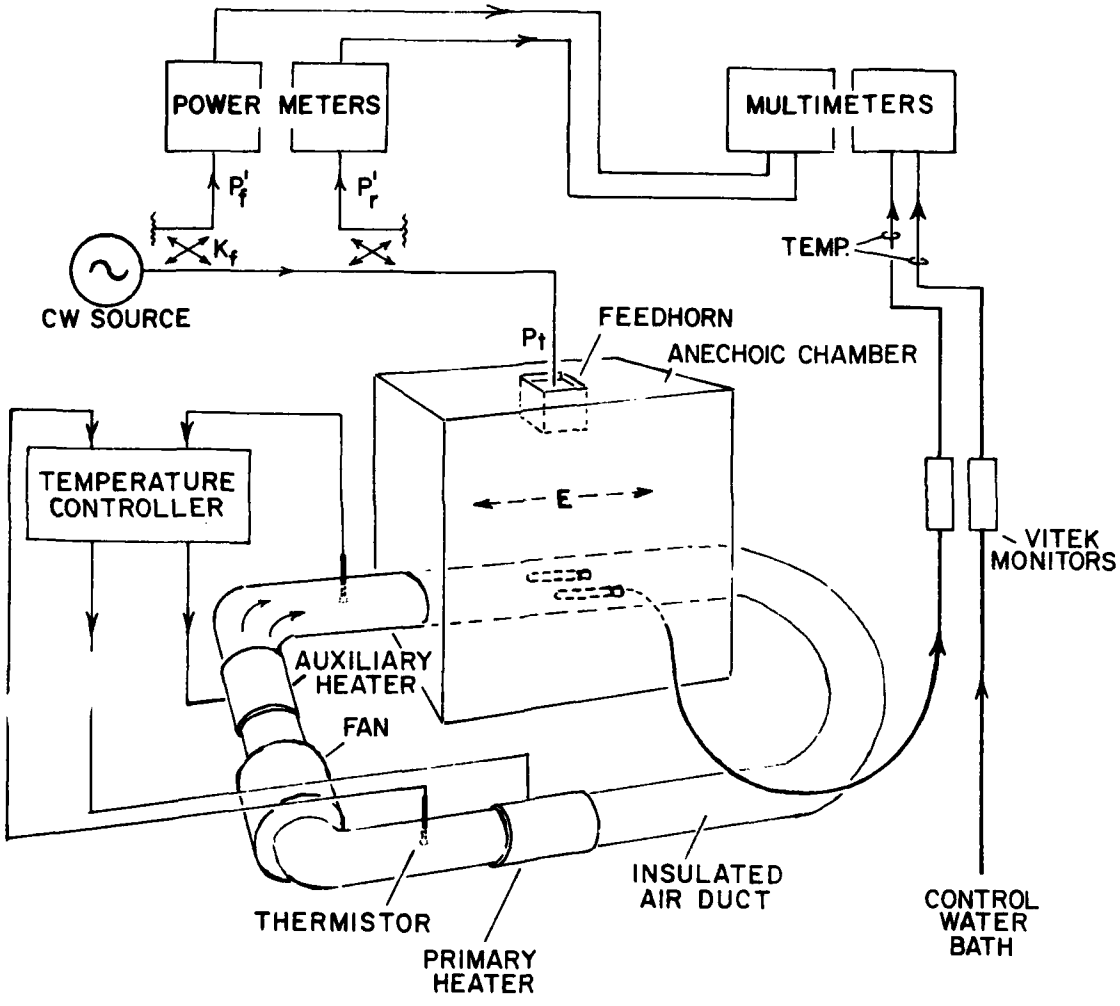

Figure 1. Schematic diagram of RF exposure system and constant-temperature-air circulator.

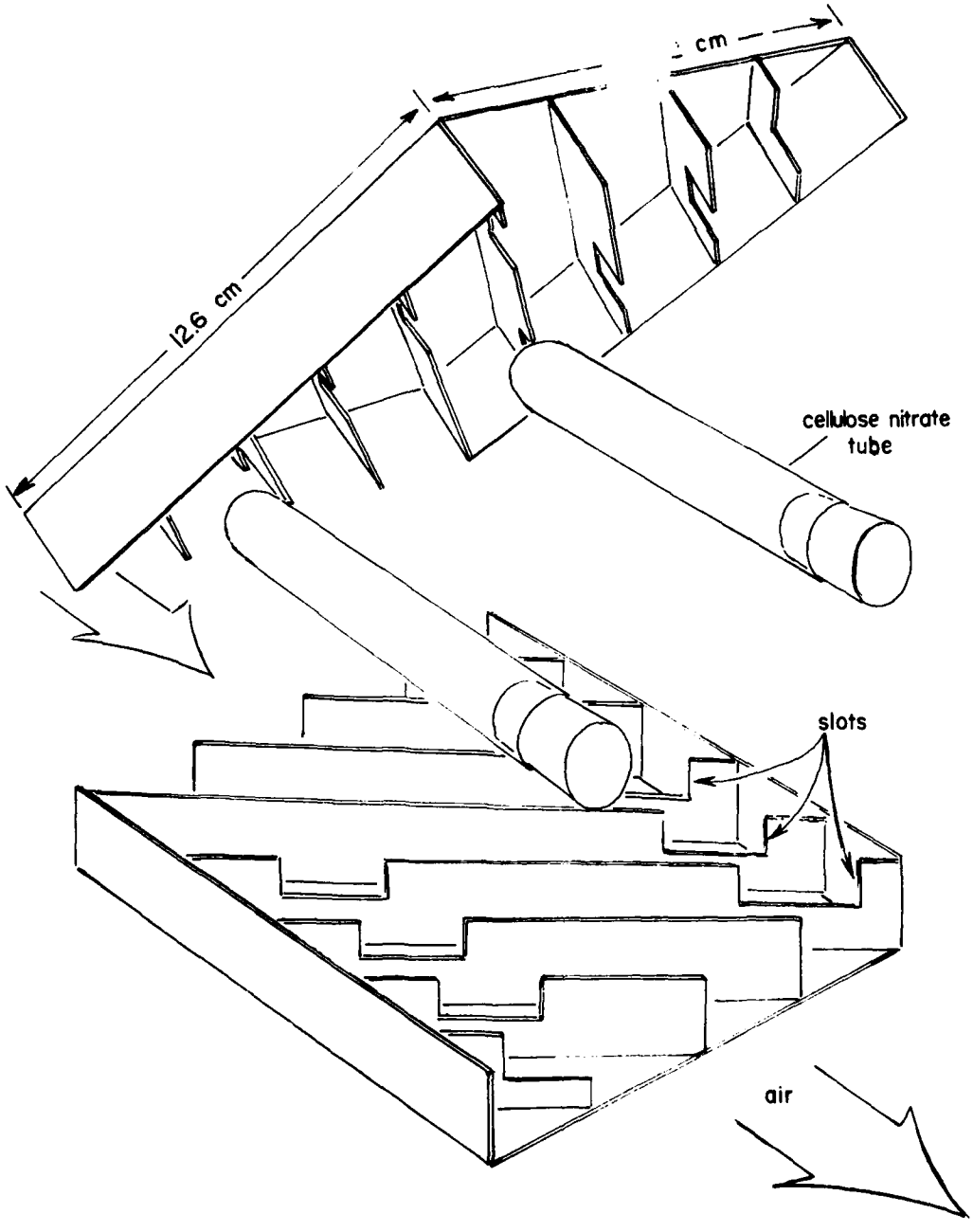

Figure 2. Exploded view of sample tube holder.

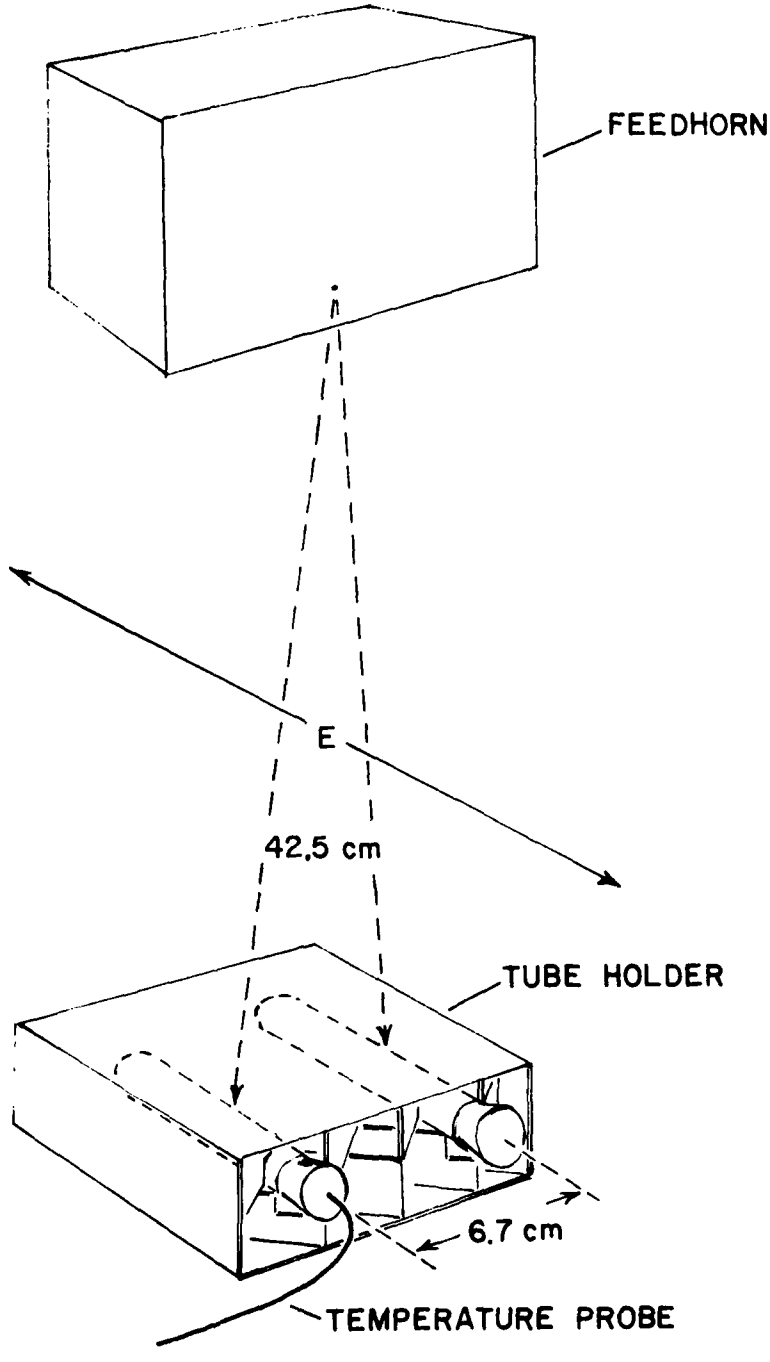

Figure 3. Position of samples in RF field.

Cell Line and Culture Conditions

A murine erythroleukemic (MEL) cell line, clone 745 (GM86), was purchased from the Institute for Medical Research, Camden, N.J. The MEL cells were grown in suspension in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Stock cultures were grown in 60- or 100-mm diameter dishes containing antibiotic-free medium and were maintained at cell densities below 10×10^5/ml to permit continuous proliferation. Incubation was at 37°C in a humidified 5% CO_2 atmosphere. Cultures used in RF exposure experiments were grown in medium supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), and 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (pH 7.3). The latter substance was added to help maintain the pH of the medium during growth outside the 5% CO_2 atmosphere. Culture vessels for these experiments were 1.6- x 10-cm cellulose nitrate tubes (Beckman) with silicone closures. The tubes were presterilized by ethylene oxide treatment. Growth of cultures was monitored with a hemacytometer.

Differentiation: Induction and Analysis

Erythroid differentiation of MEL cells was induced by incubation for 3 to 5 days in medium containing HMBA. Except where indicated, the concentration of HMBA used was 3 mM. Formation of hemoglobin, evidence of erythroid differentiation, was assessed by one or more of three tests. The first involved staining with a benzidine reagent prepared as described by Orkin et al. (26) to determine the number of cells containing hemoglobin. Under the conditions used, hemoglobin-containing cells stained dark blue with benzidine. The second test involved measurement of 415-nm absorbance, the absorbance maximum of oxyhemoglobin, by lysates prepared from the HMBA-treated cultures (9). Cells were collected from the cultures, washed with phosphate-buffered saline, and sedimented at 500 G. The washed cells were resuspended in water, 1.0 ml per 10^7 cells, subjected to three freeze-thaw cycles, and homogenized in a 2-ml Dounce homogenizer. Absorbance of the clear lysate collected by centrifugation for 10 min at 10,000 G was measured in a dual-beam spectrophotometer (model 25, Beckman) using lysate prepared from untreated cells, 10^7 cell per ml water, as a blank for comparison. The third test involved quantitation of the amount of hemoglobin present in the lysate using the colorimetric technique of Crosby and Furth (6). Quantity of hemoglobin present was expressed relative to total protein in the lysate measured by the procedure of Lowry et al. (21).

Specific Absorption Rate (SAR) and Power Density Measurements

Two methods were used for measurement of SAR. The first method, developed in consultation with Dr. G. Bertrand (personal communication, 1983), involved measurement of decay in temperature of RF-irradiated samples immediately after inactivation of RF power. Temperature equilibrium was initially established with RF power on such that

$$P_{RF} + dW/dt = 0 \qquad (1)$$

where P_{RF} was the RF power [illegible]bed by the sample, and dW/dt was the rate of heat removal by the air [illegible], close approximation to dW/dt was determined from a measurement of the [illegible]al slope of the temperature decay curve with the

RF power inactivated. Although the rate of heat removal was a function of the temperature difference between the sample temperature and the temperature of the air flow, the initial decay rate should closely approximate the cooling rate during RF irradiation, during which time the temperature remained constant. With the RF off,

$$dW/dt = (c_s m_s + Z_t)dT/dt \qquad \text{joules/sec} \qquad (2)$$

where c_s is the specific heat of the culture medium, assumed to be 4185 J/^{o}C-kg, m_s was the mass of the culture medium, Z_t was the heat capacity of the cellulose nitrate tubes, and dT/dt was the initial slope of temperature decay in ^{o}C/sec. Equation (2) was changed in sign and divided by the sample mass to calculate SAR:

$$SAR = -\frac{(c_s m_s + Z_t)dT/dt}{m_s} \qquad \text{W/kg} \qquad (3)$$

Normalized SAR was determined by dividing the SAR values by the RF power density incident on the samples. The power density was calculated by

$$P_d = \frac{0.65AP_t}{10\lambda^2 d^2} \qquad \text{mW/cm}^2 \qquad (4)$$

where A was area of the horn aperture in m^2, P_t was the input power to the horn in Watts, λ was the wavelength in meters, and d was the distance from the center of the sample to the horn aperture in meters. The power radiated, P_t, was determined from the power meter reading PM1. In referring to Figure 1,

$$P_t = (PM1)10^{(K_f - L)/10} \qquad \text{W} \qquad (5)$$

where K_f was the coupler coefficient and L was the cable loss, both in dB.

The second method of determining SAR was Dewar calorimetry. A single cellulose nitrate tube was used with special conical caps fabricated from the bottoms of other tubes used as tube closures. This was done to minimize container mass for greater accuracy in the calorimetric assessment of sample SAR. The tube with 12 ml of culture medium was placed in a Styrofoam mold in the anechoic chamber, parallel to E-field and 42.5 cm from feedhorn. After irradiation, the tube was quickly transferred to a Dewar flask (model 21F, Thermos) containing 100 ml water. Heat transferred to the water and, in turn, SAR of the sample were calculated using formulae described by Durney et al. (7).

RESULTS

Characterization of MEL Cell Growth Kinetics

Proliferation of MEL cells at 37^{o}C was monitored for 4 days using cultures maintained in 100-mm dishes with normal medium or medium containing 3 mM HMBA. Results obtained (Fig. 4) reveal that the normal medium supported exponential

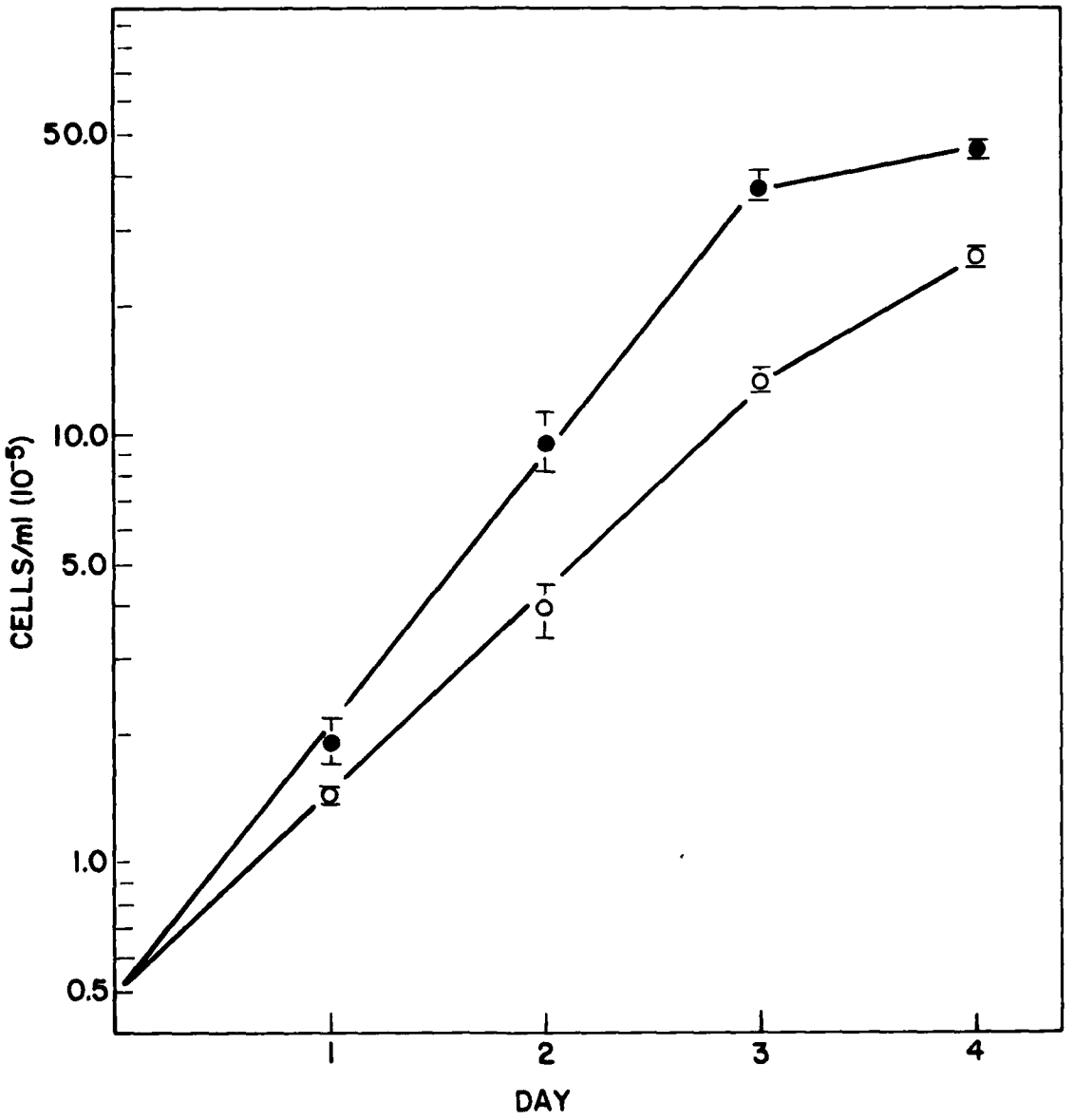

Figure 4. Growth of MEL cells in normal medium and HMBA medium. Cultures were initiated at a density of 0.5 x 10^5 cells/ml in normal medium or medium containing 3 mM HMBA. Each point and bracket represent the mean and the range of three measurements. Normal medium (●); HMBA medium (O).

growth of the MEL cultures to a density of about 3.5×10^6 cells/ml with a doubling time of about 11.8 hr. MEL cells cultured in 3 mM-HMBA medium grew at progressively slower rates. Cell doubling times were about 15.4 hr during the first 3 days of growth in 3 mM HMBA and about 24 hr during the last day of incubation. Although not shown here, we have consistently observed similar reductions in MEL cell proliferation during incubation in the presence of HMBA.

Growth of MEL cells as a function of temperature was also tested. Equal volumes of a suspension of MEL cells in normal medium were inoculated into screw-cap tubes. Three groups of tubes were immersed in separate water baths adjusted to 36.0, 37.0, and 38.0°C with thermistor temperature standard (model S-10, Thermometrics). The temperatures of the circulating water baths, monitored with Vitek 101 probes, were found to remain constant within ± 0.1°C for the 48 hr duration of the incubation. Cell densities (Table 1) increased as a function of incubation temperature with a corresponding decrease in cell doubling time. Results of this experiment suggest that MEL cells proliferate about 19% faster at 38°C than they do at 36°C.

TABLE 1. EFFECTS OF TEMPERATURE ON THE GROWTH OF MEL CELLS

Plastic tubes (16 x 125 mm) were inoculated with 8 ml of a MEL cell suspension, (0.6×10^{-5} cells/ml) in normal medium. Incubation was for 48 hr at the temperature indicated. Each cell density value is a mean ± S.E.M. for three replicate tubes.

Temperature	Final cell density (cells/ml·10^{-5})	Mean doubling time
36.0 ± 0.1°C	6.7 ± 0.6	13.9 hr
37.0 ± 0.1°C	7.1 ± 0.5	13.5 hr
38.0 ± 0.1°C	10.2 ± 0.6	11.7 hr

Response of MEL Cells to HMBA

Dose-response experiments were performed to monitor the fraction of MEL cells induced to differentiate as a function of dosage of HMBA. MEL cells suspended in medium containing HMBA, 0 to 4 mM, were inoculated into 25 cm^2 flasks and incubated for 5 days. Cultures were assayed for proliferation and differentiation. Results of one experiment, presented in Table 2, indicate that 3 mM and 4 mM HMBA induced differentiation of about 70%, or more, of the cells in the flasks. Both dosages partially inhibited proliferation. The 3 mM dosage had a less pronounced inhibition of MEL cell proliferation, however, than did the 4 mM dose. HMBA at a dosage of 2 mM induced differentiation of slightly less than half of the MEL cells while 1 mM was even less effective. The 3 mM dosage appeared the best compromise between high induction and minimal inhibition of proliferation.

TABLE 2. EFFECT OF HMBA CONCENTRATION ON PROLIFERATION AND DIFFERENTIATION OF MEL CELLS

Five sets of 25-cm^2 flasks were inoculated with 6-ml volumes of MEL cells in medium containing 0, 1, 2, 3, or 4 mM HMBA at initial densities of 0.3×10^5, 0.5×10^5, 0.75×10^5, 1×10^5, and 1.5×10^5 cells/ml, respectively. Proliferation of the cells was measured on day 3 of the incubation and erythroid differentiation was assayed on day 5. Each value is a mean of three replicate flasks.

HMBA concentration	Proliferation		Differentiation		
	Cell density (Cells/ml·10^{-5})	Doubling time (hr)	% B+[a]	A_{415}[b]	Hb[d]
0 (control)	11.0	13.8	2	--[c]	4
1 mM	14.0	14.9	19	0.02	31
2 mM	15.6	16.5	42	0.18	89
3 mM	16.6	17.7	71	0.29	137
4 mM	10.5	26.0	72	0.52	151

[a]Percent benzidine-positive cells
[b]Absorbance at 415 nm of 1 ml lysate from 10^7 cells
[c]Lysate from 10^7 untreated cells was used as blank
[d]Hemoglobin (Hb) content of lysate, µg Hb/mg total protein; lysate prepared from mature mouse erythrocytes, 16 x 10^{-12} g Hb/erythrocyte (Altman and Dittmer (1)), was used as standard for comparison.

Morphological changes were also evident in the HMBA-treated cells. Normal, untreated MEL cells possessed large nuclei and little cytoplasm (Fig. 5, views A and C). The nuclei of cells maintained for 5 days in 3 mM HMBA were condensed (Fig. 5, view B) and possessed a high affinity for Wright's stain (Fig. 5, view D). Differences in the pattern of benzidine staining of normal and HMBA-treated cells (Fig. 5, views E and F) reflect cytoplasmic accumulation of hemoglobin during the induction period.

The response of MEL cells to HMBA treatment was further characterized by results of experiments designed to monitor the kinetics of HMBA-induced erythroid differentiation. Cells were collected by centrifugation at 24-hr intervals for 5 days from MEL cultures grown in the presence of 3 mM HMBA. The color of the packed cells changed during the 5-day induction period from light amber on day 1 to slightly pink on day 3 to distinctly red on days 4 and 5. Cells collected from the cultures of one kinetics experiment were lysed and the lysates used for absorption spectra analysis. Similar lysates were prepared from circulating erythrocytes withdrawn from mature CD-1 mice. Absorption spectra of the 10,000 G supernatant fraction of these lysates are compared in Figure 6. Also shown in the figure is the absorption spectra of oxyhemoglobin. Characteristics revealed by the spectra include, firstly, a sharp peak of absorption at 415 nm in both the erythrocyte lysate and the oxyhemoglobin standard. The second major characteristic is a graduated series of 415-nm absorption peaks in the lysates of the HMBA-treated MEL cells. The height of these peaks increased with duration of exposure to the inducer. Another identical kinetics experiment was conducted and assayed in a complementary manner. Cells were collected at 24-hr intervals from HMBA-treated MEL cultures and measurements of percent benzidine-positive cells plus absorption at 415 nm were performed. Results obtained (Table 3) reveal increases in both percent benzidine-positive cells and 415-nm absorption as a function of the duration of HMBA treatment.

The final set of experiments performed to characterize the response of MEL cells to inducer involved measurement of the duration of HMBA exposure required for commitment of a large fraction of the MEL culture to erythroid differentiation. MEL cells were grown in 12 ml of 3-mM-HMBA medium for 1 to 4 days, resuspended in 4 ml normal medium (no HMBA) and the incubation continued. After incubation for 5 days total, including time incubated both in HMBA medium and in normal medium, cultures were analyzed for differentiation. Results obtained (Table 4) indicate that very few MEL cells became committed to erythroid differentiation as a result of a 24-hr exposure to HMBA. Cultivation of MEL cells for 3 days in HMBA medium and then in normal medium for 2 days resulted in almost the same degree of commitment as was achieved by a 5-day exposure to the inducer. These findings indicate that commitment of MEL cells to erythroid differentiation occurred primarily between hours 24 and 72 of HMBA exposure. Accordingly, the protocol adopted for assessing effects of RFR on erythroid differentiation (Fig. 7) involved irradiation of HMBA-treated cultures between hours 24 and 72 of the induction period followed by incubation for 2 days in normal medium.

Power Density and Field Uniformity

The broadband radiation probe was used to measure the 1180-MHz field along the 10.2- x 12.6-cm area occupied by the sample tube holder. With input power adjusted to deliver an anticipated (or calculated) power density of 22 mW/cm^2,

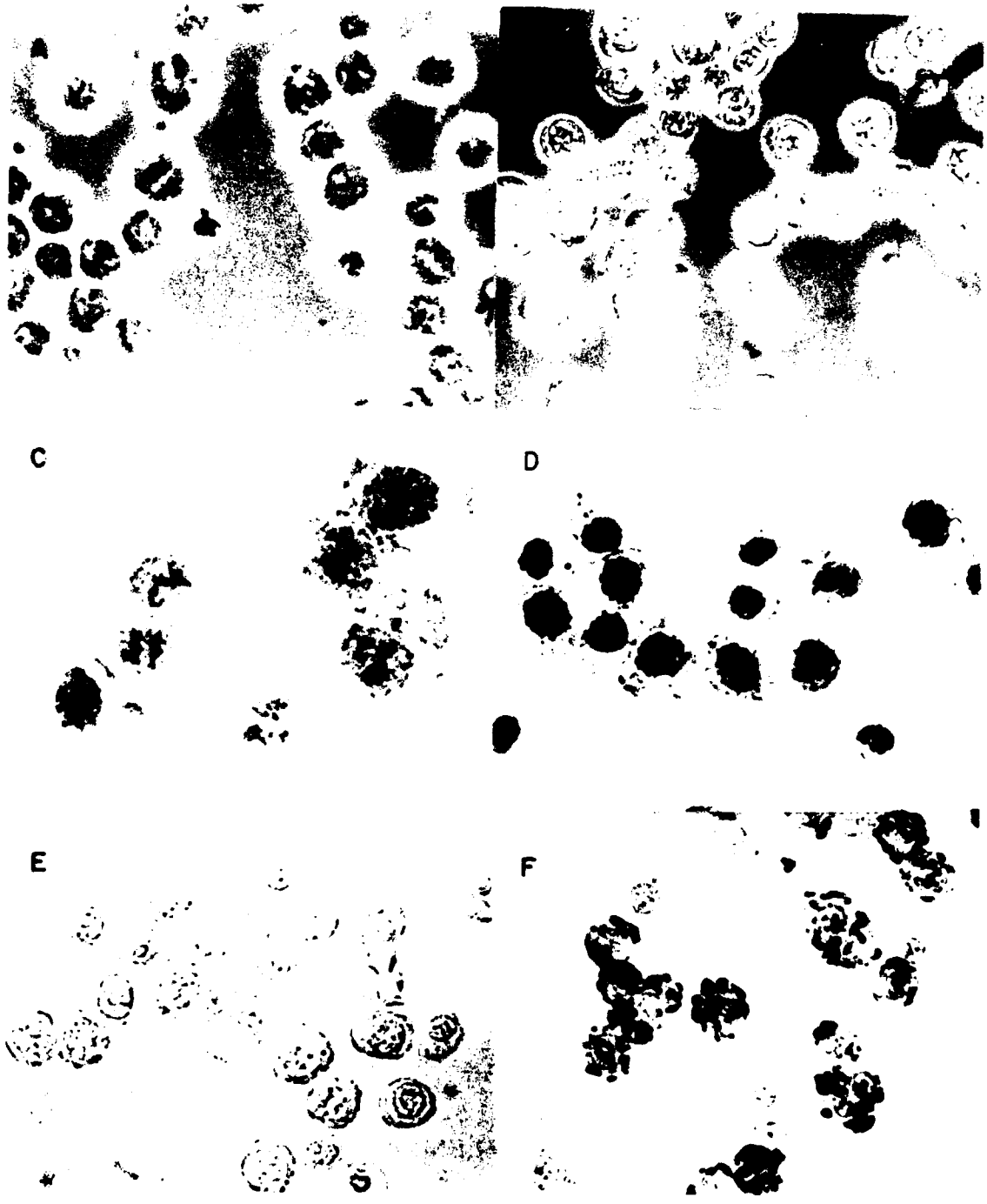

Figure 5. Micrographs of normal and HMBA-treated MEL cells. Cells were examined by phase-contrast microscopy (A and B) and by bright-field microscopy following addition of Wright's stain (C and D) or benzidine reagent (E and F). Untreated control cells are shown in views A, C, and E; Views B, D, and F show cells treated with 3-mM HMBA for 5 days.

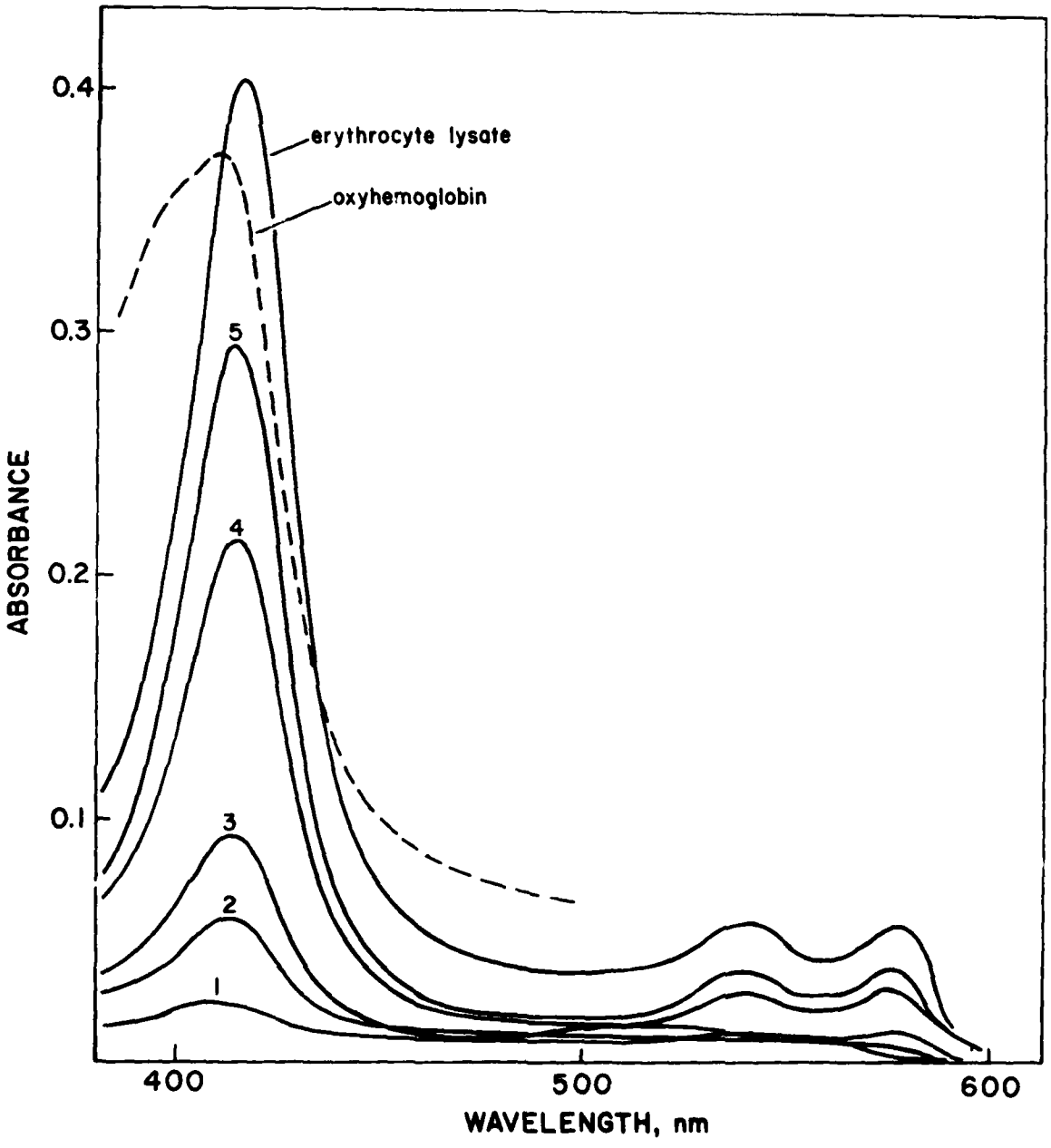

Figure 6. Absorption spectra of lysates from MEL cells grown in HMBA. Cells collected from 3-mM-HMBA-treated cultures were resuspended in water, 1 ml/10^7 cells, for lysis. Lysates were prepared from mature mouse erythrocytes, 0.4 x 10^7 cells/ml water, for comparison. Readings were taken on a dual-beam spectrophotometer. Lysate from 10^7 untreated cells was used for an absorbance blank. Numbers on lysate scans refer to days in HMBA.

TABLE 3. KINETICS OF HMBA-INDUCED DIFFERENTIATION OF MEL CELLS

Five sets of 100-mm dishes were inoculated with 8-ml volumes of MEL cells in medium containing 3-mM HMBA at initial densities of 8×10^5, 6×10^5, 4×10^5, 2×10^5, and 1×10^5 cells/ml for incubations of 1, 2, 3, 4, and 5 days, respectively. Upon completion of incubation, cells were collected for analysis of differentiation. Cells from untreated control cultures were collected for comparison. Each value is a mean ± S.E.M. for three replicate dishes.

HMBA Treatment (days)	Differentiation	
	% B+[a]	A_{415}[b]
0 (control)	1 ± 0.4	--
1	1 ± 0.4	0.004 ± 0.002
2	16 ± 2	0.02 ± 0.01
3	30 ± 3	0.09 ± 0.005
4	62 ± 1	0.23 ± 0.02
5	71 ± 7	0.33 ± 0.03

[a]Percent benzidine-positive cells
[b]Absorbance at 415 nm of 1 ml lysate from 10^7 cells

TABLE 4. COMMITMENT OF MEL CELLS TO ERYTHROID DIFFERENTIATION AS A FUNCTION OF TIME IN HMBA

Five sets of 100-mm dishes were inoculated with 9-ml volumes of MEL cells in 3-mM HMBA medium at initial densities of 6×10^5, 2.5×10^5, 1.2×10^5, 0.6×10^5, and 0.5×10^5 cells/ml for incubations of 1, 2, 3, 4, and 5 days, respectively. Cells were then resuspended in 3 ml of normal medium (no HMBA), placed in 60-mm dishes, and the incubations continued for 1 to 4 days. After incubation for 5 days total, cultures were assayed for differentiation. Each value is a mean ± S.E.M. for three replicate dishes.

Incubation: medium and duration		Differentiation	
HMBA medium	Normal medium	% B+[a]	A_{415}[b]
--	5 days	2 ± 0.3	--
1 day	4 days	17 ± 2	0.06 ± 0.02
2 days	3 days	40 ± 1	0.24 ± 0.05
3 days	2 days	68 ± 1	0.52 ± 0.04
4 days	1 day	73 ± 3	0.46 ± 0.03
5 days	--	75 ± 1	0.43 ± 0.01

[a]Percent benzidine-positive cells
[b]Absorbance at 415 nm of 1 ml lysate from 10^7 cells

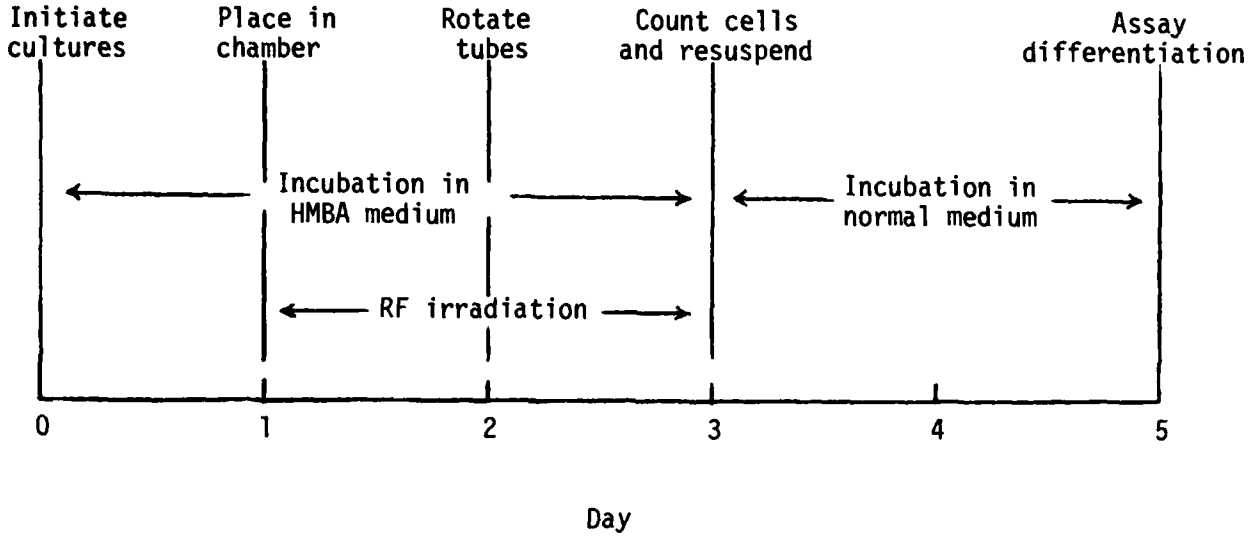

Figure 7. Protocol for assessing effects on proliferation and differentiation of HMBA-treated MEL cells. Cultures (12 ml) were initiated at a density of 0.5×10^5 cells/ml in 3-mM-HMBA medium. Cultures were incubated for 24 hr, then placed in either the RF-exposure chamber or a circulating water bath and incubation resumed. Positions of the two irradiated tubes were exchanged at the midpoint of the 48-hr exposure period. Cells were counted at day 3 for determination of growth rate and then resuspended in normal medium (4 ml) for 2 additional days incubation before assessment of erythroid differentiation.

the power densities measured with the probe ranged from 24.7 mW/cm^2 at the center to 22.1 mW/cm^2 at the air-inflow end of the tube holder. These data reveal, firstly, that the power density at the center, the point of maximum power density, was about 10% higher than the minimum within the area surveyed. Secondly, the data indicate that the measured values, average 23.4 mW/cm^2, were about 6% higher than the calculated value, 22 mW/cm^2. Power densities measured at the two lower exposure levels, nominally 5.5 and 11 mW/cm^2, also average about 6% higher than the calculated values.

Sample SAR

SAR measurements were made by the temperature decay technique with one or two tubes, each containing 12 ml of culture medium, in the anechoic exposure chamber. The curves in Figure 8 are typical of the temperature decay data recorded with two tubes in the chamber and the probe of the Vitek 101 meter inserted through the silicone closure of one of the tubes. Temperature equilibrium was established with RF power adjusted to 5.5, 11, or 22 mW/cm^2. After equilibrium was attained, usually 15 to 20 min, RF power was quickly dropped to 0 mW/cm^2. The slope of the line fitted to the recordings obtained during the first 60 sec after terminating RF power was used to calculate heat removed from the sample by the air flow, which was then used to calculate sample SAR. The figure reveals that the slope of the temperature decay was directly proportional to the incident power density.

The slope of the temperature decay that followed 11-mW/cm^2 irradiation of the specific sample represented in the figure was -0.00854 ^{o}C/sec. The mass of that sample was 0.012 kg. The heat capacity of the cellulose nitrate tube was estimated to be 0.6 times that of an equivalent amount of water, or 6 J/^{o}C. Using Equation (3) in TECHNICAL APPROACH, the SAR of this sample was calculated as follows:

$$\begin{aligned} SAR &= -\frac{(c_s m_s + Z_t)dT/dt}{m_s} \quad W/kg \\ &= -\frac{[(4185)(0.012) + 6](-0.00854)}{0.012} \quad W/kg \\ &= 40.01 \quad W/kg \end{aligned} \tag{6}$$

This value plus those from two other measurements yielded a mean value of 36.62 W/kg for samples exposed to 11 mW/cm^2. Calculations similar to the above were performed to determine sample SAR at the other two RF-exposure levels tested. The SAR values measured by the temperature decay method were directly proportional to the RF-power density incident on the samples, as shown in Figure 9. The normalized SAR with two tubes in the chamber was determined from these measurements to be about 3.35 W/kg per mW/cm^2.

The SAR measurements by the temperature decay technique were also made with only one sample tube in the exposure chamber. The resultant SAR values calculated for two separate exposures at 11 mW/cm^2 were 56.4 and 60.2 W/kg, or a mean of 58.3 W/kg.

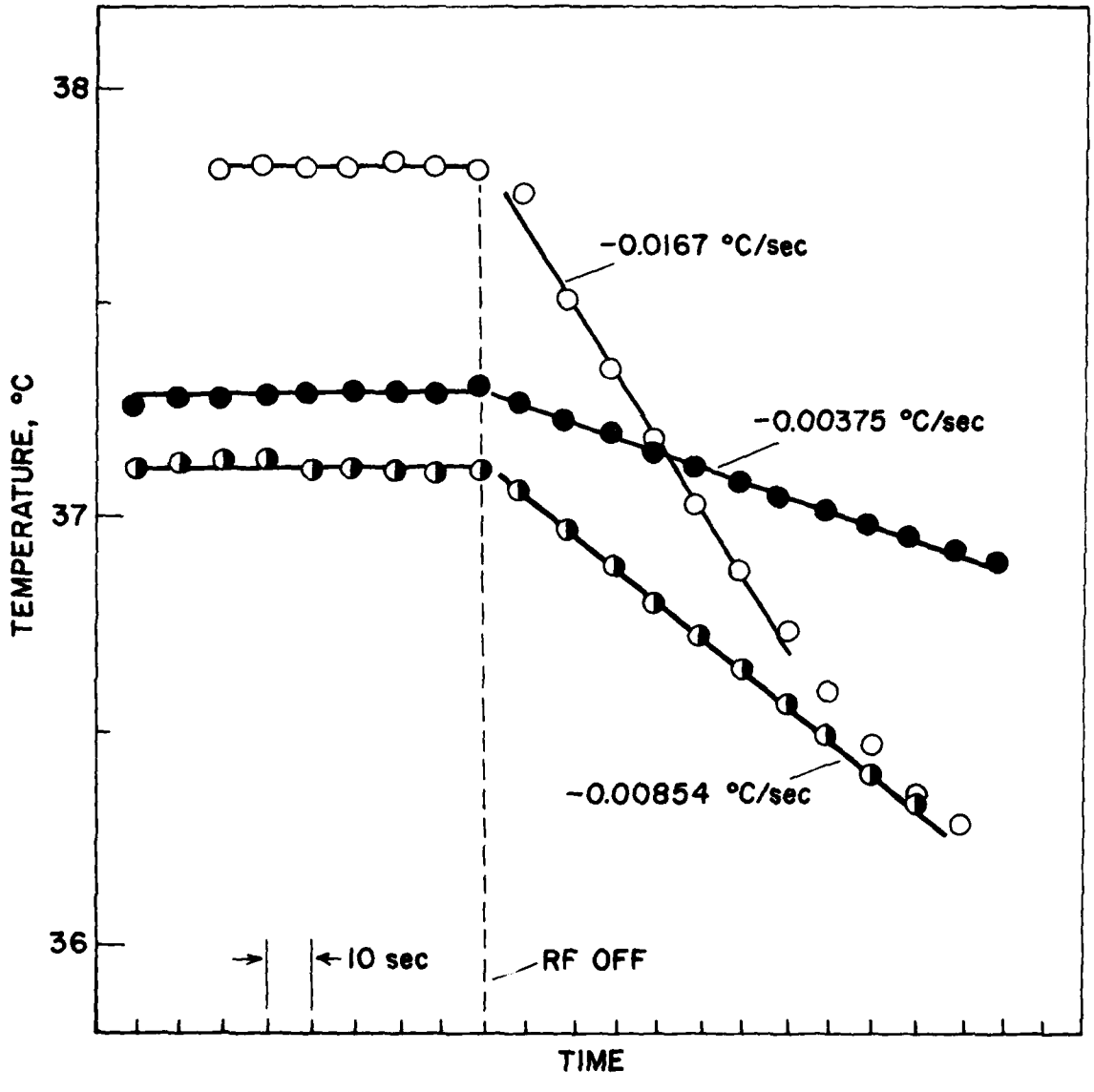

Figure 8. Decay of sample temperatures following 1180-MHz irradiation. Two 12-ml cultures were exposed to 1180-MHz field intensities of 5.5 (●), 11 (◑), and 22 mW/cm^2 (○) and circulating-air temperatures adjusted to maintain constant temperature. The slope of the temperature decay following cessation of irradiation was used to determine SAR.

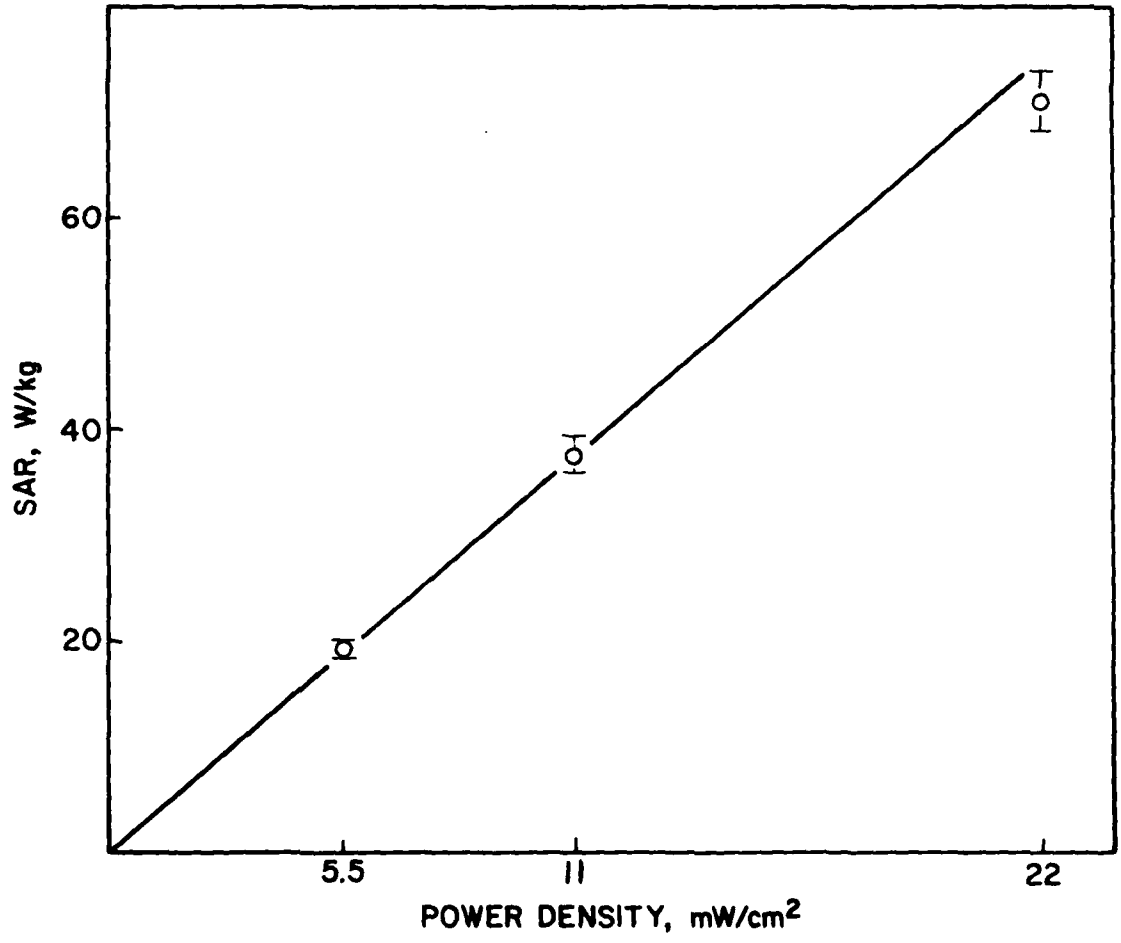

Figure 9. Relationship between sample SAR and incident power density. SAR values were determined from temperature decay following irradiation. Open points and vertical brackets represent the mean and the range of three measurements at each power density.

Dewar calorimetry was performed to test the validity of the SAR values measured by the temperature decay technique. A single tube containing 12 ml of culture medium was placed in the Styrofoam mold, positioned in the anechoic chamber parallel to the E-field and 42.5 cm from the center of the feedhorn aperture, and irradiated at 11 mW/cm^2. The mean ± S.E.M. of SAR values calculated from four separate calorimetric measurements was 63.9 ± 6.9 W/kg. Comparison of this value with the 58.3 W/kg result by temperature decay suggests that the SAR estimates obtained by the temperature decay technique were about 9% lower than that actually absorbed.

Culture Temperature During Irradiation

Numerous pretests were conducted with various tube configurations and air-circulator ducts in an attempt to develop a system that would satisfy the following criteria: support of rapid cell growth; maintenance of multiple tubes at an identical, constant temperature; and exposure of multiple tubes to the same RF field strength. Five different sample holders were tested including a holder for four vertically positioned tubes plus four others designed for two or three horizontally positioned tubes. Vertical positioning of tubes was abandoned because it did not support rapid cell growth. Usage of three horizontally positioned tubes also proved unsatisfactory. The center tube was consistently 0.4 to 1.5°C warmer than the two outer tubes in irradiation pretests conducted at 11 and 22 mW/cm^2. The only configuration that satisfied all three criteria involved usage of two horizontally positioned tubes. Two tubes in the holder designed for a crisscrossed air flow remained at the same temperature (within ± 0.1°C) during irradiation at 22 mW/cm^2 as determined by simultaneous recordings of temperatures in the two tubes using the Vitek 101 meters.

The two-tube configuration was used in all the subsequent tests of RF effects on differentiation with tube temperature monitored during each exposure. This was accomplished by insertion of a Vitek probe through the silicone closure used for one tube of each irradiated pair. The probe of a second Vitek meter was inserted in the water bath used for incubation of the control cultures. Temperatures recorded for a tube exposed to 22 mW/cm^2 are compared in Figure 10 with those recorded for the control water bath. As shown, both the irradiated and the control samples remained at essentially the same temperature, 37.4°C, during the 48-hr exposure period. The slight dip in temperature at 24 hr was associated with rotation of the tubes. These recordings are typical of those obtained in the other experiments of this study.

Proliferation and Differentiation of MEL Cells During Irradiation

Twelve experiments were conducted to monitor the growth and differentiation of HMBA-treated MEL cells exposed to various levels of 1180-MHz radiation. Results of four replicate exposures at a power density of 5.5 mW/cm^2 are presented in Table 5. Results obtained for cultures irradiated at 11 mW/cm^2 are given in Table 6, and results from the 22-mW/cm^2-exposures are in Table 7.

An overview of the data presented in these three tables reveals that the cell-doubling times of the control and irradiated cultures ranged from 15 to 20 hr. The percentage of benzidine-positive cells ranged from 20 to 87% with

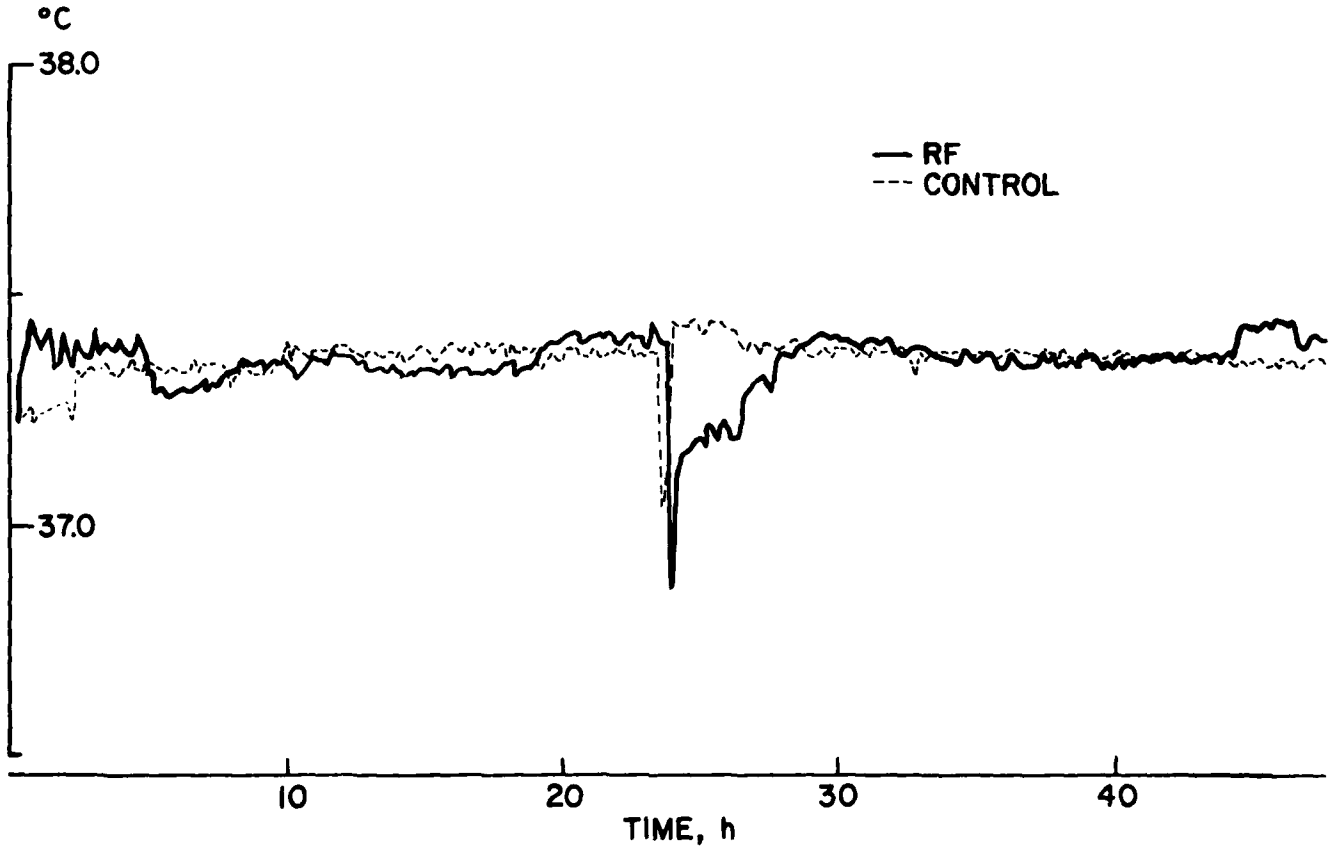

Figure 10. Comparison of irradiated-tube temperature and control-tube temperature during exposure period. Irradiated cultures were exposed at 22 mW/cm^2. Temperatures were recorded at 10-min intervals with Vitek meters.

TABLE 5. PROLIFERATION AND DIFFERENTIATION OF MEL CELLS EXPOSED TO 5.5 mW/cm^2

Experiments were initiated by suspending MEL cells, 0.5 x 10^5 cells/ml, in medium containing 3-mM HMBA. Each replicate experiment included two unirradiated control cultures (C) and two 1180-MHz-exposed cultures (RF). Cell doubling time was determined at day 3 and differentiation at day 5 in each experiment.

Replicate No.	Culture sample	Doubling time (hr)	Differentiation	
			% B+[a]	Hb[b]
I_a	C_1	16.1	69	57
	C_2	17.6	74	37
	RF_1	16.6	76	32
	RF_2	17.3	72	35
I_b	C_1	15.5	71	38
	C_2	15.4	69	41
	RF_1	16.3	65	45
	RF_2	15.3	71	47
I_c	C_1	17.1	56	52
	C_2	15.8	20	43
	RF_1	18.1	32	34
	RF_2	16.8	58	57
I_d	C_1	17.8	57	44
	C_2	18.1	63	38
	RF_1	18.2	64	47
	RF_2	20.1	53	45

[a]Percent benzidine-positive cells
[b]Hemoglobin content, µg/mg total protein in lysate

TABLE 6. PROLIFERATION AND DIFFERENTIATION OF MEL CELLS EXPOSED TO 11 mW/cm^2

Replicate No.	Culture sample	Doubling time (hr)	Differentiation	
			% B+[a]	Hb[b]
II_a	C_1	15.2	59	71
	C_2	14.9	55	84
	RF_1	16.1	56	59
	RF_2	14.5	56	97
II_b	C_1	15.4	66	91
	C_2	15.1	72	92
	RF_1	15.3	87	57
	RF_2	16.2	63	96
II_c	C_1	17.1	67	46
	C_2	18.2	67	56
	RF_1	18.1	70	45
	RF_2	16.5	79	44
II_d	C_1	14.6	75	53
	C_2	15.5	82	65
	RF_1	15.8	83	76
	RF_2	15.9	79	69

[a]Percent benzidine-positive cells
[b]Hemoglobin content, µg/mg total protein in lysate

TABLE 7. PROLIFERATION AND DIFFERENTIATION OF MEL CELLS EXPOSED TO 22 mW/cm^2

Replicate No.	Culture sample	Doubling time (hr)	Differentiation	
			% B+[a]	Hb[b]
III_a	C_1	15.1	80	66
	C_2	15.8	82	63
	RF_1	15.8	71	48
	RF_2	15.5	72	56
III_b	C_1	15.6	75	87
	C_2	16.5	72	64
	RF_1	16.1	81	84
	RF_2	16.8	70	88
III_c	C_1	19.5	59	58
	C_2	19.4	51	41
	RF_1	16.3	50	63
	RF_2	15.9	49	60
III_d	C_1	17.0	40	59
	C_2	17.4	54	58
	RF_1	16.4	47	51
	RF_2	16.1	51	53

[a]Percent benzidine-positive cells
[b]Hemoglobin content, µg/mg total protein in lysate

most of the values falling between 50 to 80%. The hemoglobin content of the lysates prepared from the experimental, HMBA-treated cultures ranged from 32 to 96 μg/mg total lysate protein. The hemoglobin content of lysates prepared from normal (-HMBA) cultures was also measured. The mean of values obtained for five untreated cultures was 2.6 μg/mg total lysate protein. In contrast, the hemoglobin content of a lysate prepared from mature mouse erythrocytes was found to be 610 μg/mg total lysate protein.

All cultures used in the twelve irradiation experiments assayed were established at the same initial density, 0.5×10^5 cells/ml, and exhibited similar growth kinetics with doubling times ranging from about 15 to 20 hr. Replicates were, thus, pooled and treatment means calculated in an attempt to compare effects of the three different irradiation levels on the growth and the differentiation of the HMBA-treated MEL cells. The treatment means calculated as described are compared in Table 8. The results summarized in the table indicate no significant effect of any of the three power densities tested on the rate of proliferation of the MEL cells, the percent of the cells that were stained with benzidine, or the amount of hemoglobin present in lysates prepared from the cells.

DISCUSSION

Organisms are particularly vulnerable to known chemical and physical hazards during periods of growth and differentiation. Chemical induction of tumors in the developing rat brain (12) and the regenerating liver of adult rats (5), for example, are correlated with rapid cellular proliferation in those organs. In a similar manner, the stages of early embryo development, periods characterized by commitment of progenitor cells to specific modes of differentiation, are also very sensitive to agents that alter nucleic acid integrity (3). It is not surprising that many investigations of bioeffects of low-level RF radiation, an unproven hazard, have focused on developing systems. This investigation was initiated to assess RF effects on cells undergoing growth and differentiation in a highly controlled environment in vitro. The investigation was unique because of both the tight control of exposure temperature and the cell system used.

The MEL cell was chosen for this project because of the extensive body of literature characterizing its pattern of differentiation. The initial phases of the project consisted of familiarization with the cell and development of a suitable protocol for assessing RF effects on MEL differentiation. MEL response to two different chemical inducers, dimethylsulfoxide (DMSO) and HMBA, was tested. HMBA proved less toxic to the cells and was adopted for use.

It is generally recognized that erythroid differentiation of MEL cells follows a latent period that varies with culture conditions and type of inducer added (22). Gusella et al. (11) reported a latent period of 12 to 18 hr before any committed cells appeared in DMSO-treated cultures. The latter group found the proportion of committed cells to increase sharply beginning at about 24 hr. We observed a similar lag following addition of HMBA. The percentage of committed cells was about 17%, 40%, and 68% after incubation for 1, 2, and 3 days, respectively, in 3 mM HMBA. These findings plus the observation of about 75% committed cells in cultures incubated for 5 days with HMBA suggested that most of the molecular events of differentiation, or commitment, occurred between

TABLE 8. COMPARISON OF RF POWER DENSITY EFFECTS ON PROLIFERATION AND DIFFERENTIATION OF MEL CELLS

Data presented in Tables 5, 6, and 7 were pooled and mean values computed for control (C) and irradiated (RF) cultures. Each value is the mean ± S.E.M. of eight observations. Means were compared by a two-tailed t-test. None of the means were found to differ.

Power density (mW/cm^2)	Culture condition	Doubling time (hr)	Differentiation	
			% B+[a]	Hb[b]
5.5	C	16.7 ± 0.4	60 ± 7	44 ± 3
	RF	17.3 ± 0.6	61 ± 5	43 ± 3
11	C	15.8 ± 0.5	68 ± 3	70 ± 7
	RF	16.1 ± 0.4	72 ± 5	68 ± 8
22	C	17.0 ± 0.6	64 ± 6	62 ± 5
	RF	16.1 ± 0.2	62 ± 5	63 ± 6

[a]Percent benzidine-positive cells
[b]Hemoglobin content, µg/ml total protein in lysate

day 1 and day 3. We predicted that to be the duration when RF exposure might have greatest effect.

The rationale for three other aspects of the protocol requires elaboration. These include measurement of proliferation on day 3, the 2-day incubation in normal medium that followed growth in HMBA medium, and the three-fold concentration of cells for the latter incubation. Day 3 was chosen for measurement of proliferation because cultures were, as shown in Figure 4, still in the exponential-growth phase. A two-day, follow-up incubation in normal medium was required for expression of erythroid differentiation. Comparison of results in Tables 3 and 4 reveals that even though there were 68% committed cells at day 3, only 30% of the day 3 cells contained enough hemoglobin to react with benzidine. The two extra days of incubation, an expression period, permitted accumulation of hemoglobin in the committed cells. The three-fold concentration during resuspension was included as a means of inhibiting continued proliferation of uncommitted cells in the cultures during the expression period. Gusella et al. (11) found committed cells were capable of only one additional division after 3 days in inducer whereas the uncommitted cells continued to proliferate when resuspended at low cell density. Figure 4 indicates that cell growth ceased at a density of 3-4 x 10^6 cells/ml. Three-fold concentration of the day 3 cultures would place them at that density.

Results of investigations based on hypotheses of RF interaction with biologic entities by an athermal, resonant mechanism can only be meaningful when temperature artifacts are rigorously precluded. A considerable portion of this investigation involved development of a system for controlling the temperature of the irradiated cultures. The constant-temperature-air circulator and the specially constructed tube holder permitted maintenance of the irradiated and control cultures at essentially the same temperature, within ± 0.1°C, as determined by the Vitek meters. A simple test was performed to determine if recorded temperatures, such as those shown in Figure 10, were influenced by RF field interaction with the temperature probe. Temperatures of samples in a 22 mW/cm^2 field were monitored at 10-sec intervals. Cessation of RF power caused no instantaneous changes in the ple temperature recordings thereby demonstrating that the temperatures recorded were valid.

Minimal oscillation in the recording of the temperature of the irradiated samples (Fig. 10) plus rapid temperature decline following irradiation (Fig. 8) reflect efficient heat removal by the circulating air. Incidents of failure of various components of the air circulator or the control water bath resulted in termination of experiments on three separate occasions. One such failure, occurring in the air-circulator fan, resulted in cessation of air flow. The temperature of the irradiated sample, 5.5 mW/cm^2, increased within 1 hr to 52°C before the failure was noticed. Such findings might raise concerns about the significance of reported RF effects on mammalian cultures not maintained at rigorously controlled temperatures (4). Use of circulating air to regulate temperature introduced a small technical problem. We found that the cellulose nitrate tubes used for culturing were somewhat permeable to CO_2 and that the flowing air hastened the CO_2 removal resulting in the medium becoming slightly alkaline. The problem was remedied by addition of a non-volatile buffer, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, to the medium.

The frequency that was initially elected for study was 1200 MHz. The CW source was found to be erratic, however, at that frequency. The reflected

power at 1200 MHz was about 10% of the input power resulting in excessive plate current and automatic shutdown of the source after 30 to 60 min of operation at the power level required for 22 mW/cm^2 (approximately 200 W). Adjustment of the frequency to 1180 MHz reduced the reflected power to less than 1% of the forward power. This permitted the source to run continuously for several days at 22 mW/cm^2 without incident.

The thin-walled, cellulose nitrate tubes were chosen for culturing, rather than vessels such as plastic dishes or T flasks, because of their low RF absorption and their simple geometry. The spheroid-like shape of cultures in stoppered tubes simplified determination of sample SAR. The normalized SAR measured for 1180-MHz-irradiated tubes containing 12-ml cultures was 3.35 W/kg per mW/cm^2. This was about 7-fold higher than would be predicted for spheroidal samples by the formula

$$SAR \approx \sigma E_0^2 \left[\frac{(1/L)^2}{\varepsilon_1^2 + \left(\frac{\sigma}{\omega \varepsilon_0}\right)^2} + \frac{0.4\ \pi\ (\text{area})}{\lambda^2} \right] X \frac{\text{vol}}{\text{mass}} \qquad (7)$$

adapted by Marshall and Brown (23) from Lin et al. (17), where σ = conductivity = 1 S/m, E = electric field strength, 1/L = shape factor = 30.3, for 1.6 x 10 cm tubes, ε_1 = dielectric constant = 80, ε_0 = 8.854 x 10^{-12} F/m, ω = 2π·frequency, λ = wavelength, and (area) = cross-sectional area normal to wave direction. The normalized SAR value calculated for the cultures was 0.46 W/kg per mW/cm^2 using σ = 1 S/m and ε_1 = 80. Comparisons of SAR values calculated by the same formula for spheroidal samples exposed to 200- to 400-MHz radiation were very similar to measured values. The high SAR values measured in this project appeared due to the relationship between the 9-cm length of the mass of culture medium and the wavelength of the exposure frequency (λ/2 is 13 cm at 1180 MHz). This was close to a resonant frequency.

The similarity of the SAR values determined by calorimetry and by the temperature-decay method suggests that the latter measurements were accurate. We believe the most likely source of error in our measurements was the estimate of the specific heat of the cellulose nitrate (Z_t of equation 3, TECHNICAL APPROACH), assumed to be 0.6 times that of an equivalent amount of water. A 100% error in Z_t would, however, change the result only 10%. It should also be noted that the silicone stoppers were not used as tube closures during calorimetry. The stoppers were replaced by special conical caps fabricated from the bottoms of other tubes to minimize container mass during the calorimetric measurement of sample SAR.

The irradiated cultures absorbed moderately high RF dosages. The SAR values measured for exposures at 5.5, 11, and 22 mW/cm^2 were 18.5, 37, and 74 W/kg, respectively. The two-day exposures at these levels had no significant effect, however, on the rate of growth of the MEL cells. There was also no significant effect on erythroid differentiation, as determined both by the percentage of hemoglobin-containing cells present in the HMBA-treated cultures and by measurement of hemoglobin content of lysates prepared from the cultures.

The lack of any detectable differences between the control cultures and the irradiated cultures is particularly striking in view of the magnitude of the

dosages of RF absorbed, ranging from 46- to 185-fold higher than the limit proposed by Subcommittee C95.4 of the American National Standards Institute. These findings suggest that long-term exposures to continuous wave frequencies identical or similar to those used here do not constitute a hazard to the growth or differentiation of mammalian cells. This does not, however, rule out the possibility of developmental effects by pulsed waves or lower frequency radiation. Athermal interaction of RF photons with biologic systems does appear to occur as evidenced by altered activity of an immobilized enzyme exposed to 400-MHz radiation (14) and by 600-MHz-induced resonance of dry DNA (19). It remains to be determined, however, if such effects at the molecular level will be expressed at the cell or whole animal level.

CONCLUSIONS

Cultures of HMBA-treated MEL cells rigorously maintained at 37.4°C during exposure to 1180-MHz CW radiation at SAR levels of 18.5, 37, and 74 W/kg showed no evidence of reduced cell proliferation. The RF exposures also had no effect on the number of cells in the cultures that underwent erythroid differentiation as determined by reaction with benzidine. And finally, the RF exposures had no effect on the amount of hemoglobin produced by the HMBA-treated cultures. The CW frequency studied in this investigation does not, in view of proposed exposure limits, appear hazardous to growth and development of mammalian cells.

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